

## Antibiotic Disk Susceptibility Tests with *Neisseria gonorrhoeae*

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We have used a standardized disk diffusion procedure to test the susceptibility of 892 isolates of *Neisseria gonorrhoeae* to six antibiotics. The disk diffusion test described is a modification of the disk test of Bauer, Kirby, Sherris, and Turck, which is widely used for susceptibility testing of fast-growing, aerobic pathogens. With all six antibiotics (penicillin, ampicillin, tetracycline, erythromycin, chloramphenicol, and cephaloridine), disk test results were found to be reproducible and correlated well with the minimal inhibitory concentration as determined by an agar dilution procedure. The coefficients of correlation ranged from -0.79 with cephaloridine to -0.93 with penicillin. The technique described for disk susceptibility testing of gonococci can be used both for research and clinical purposes.

For at least the past two decades, the resistance of gonococci to a number of antibiotics has steadily increased (24). In 1944, almost all strains of *Neisseria gonorrhoeae* were inhibited by 0.01  $\mu\text{g}$  of penicillin per ml (22). Today, a significant number of strains require at least 1.0  $\mu\text{g}/\text{ml}$  for inhibition (17). This increased resistance has been documented with other antimicrobials as well. Many gonococcal strains are resistant to streptomycin (26) and to sulfonamides (4). Resistance to tetracycline (26) and erythromycin (20) has also increased, especially in the past 10 years.

Currently, the United States Public Health Service (USPHS) recommends four antibiotics (penicillin, ampicillin, tetracycline, and spectinomycin) for use in the treatment of gonorrhea (2). The recommended dosages of these antibiotics are now close to the maximum that can be administered on an outpatient basis (10, 11, 24, 26). This means that future increased drug resistance can not be countered by further increases in the amount of antibiotics prescribed.

In this event it may become advantageous to employ susceptibility tests as a guide to appropriate therapy. The disk technique of Bauer et al. (1) has become widely used for susceptibility testing of fast-growing, aerobic pathogens. It offers simplicity and economy in comparison with an antibiotic dilution procedure. However, no disk diffusion technique is currently accepted for susceptibility testing of gonococci (1).

We have been interested in the antibiotic resistance of *N. gonorrhoeae* for some time. In our laboratory, we have found a disk technique to be useful in studies of gonococcal antibiotic resistance. This report describes the disk test we have used and presents the correlation with the minimal inhibitory concentration for six antibiotics.

### MATERIALS AND METHODS

**Cultivation of *N. gonorrhoeae*.** A total of 892 strains of gonococci, from the Philadelphia Health Laboratory, were isolated on Thayer-Martin medium (25) by incubation for 24 to 48 h at 35 C under 5% carbon dioxide. Typical, oxidase-positive colonies, consisting of gram-negative diplococci, were confirmed as *N. gonorrhoeae* by sugar fermentation or by the fluorescent antibody procedure.

**Antibiotics.** Penicillin G, tetracycline, erythromycin, chloramphenicol, cephaloridine, and ampicillin were obtained commercially as diagnostic powders for susceptibility testing. Antibiotic sensitivity disks for the above antibiotics were purchased from Baltimore Biological Laboratories. The sensitivity disks used were penicillin (10 U), tetracycline (30  $\mu\text{g}$ ), erythromycin (15  $\mu\text{g}$ ), chloramphenicol (30  $\mu\text{g}$ ), cephaloridine (30  $\mu\text{g}$ ) and ampicillin (10  $\mu\text{g}$ ).

**Susceptibility testing.** The agar dilution method used to establish a minimal inhibitory concentration (MIC) was based on the technique previously described (6). The details are as follows. Isolates were subcultured on GC agar (Difco GC medium base, hemoglobin, and 1% supplement B) for approximately 18 h. (The inclusion of hemoglobin was optional.) By using a sterile swab, 5 to 10 colonies were suspended in 5 ml of unsupplemented GC broth (Difco GC

medium base, minus starch and agar). The suspension was agitated vigorously by blending in a Vortex mixer in order to break up the clumps of bacteria. The suspension was diluted with GC broth to contain approximately  $5 \times 10^7$  colony-forming units per ml, as judged by a barium sulfate standard (1). Twofold serial dilutions of the desired antibiotic were prepared in sterile, distilled water and incorporated into GC agar (20 ml per plate). Approximately 0.05 ml of the bacterial suspension was placed onto each plate in the series. Plates were incubated at 35 C under 5% carbon dioxide for 48 h. The MIC was accepted as the lowest concentration of antibiotic giving complete inhibition of growth.

Disk susceptibility testing of gonococcal isolates was performed essentially by the method of Bauer et al. (1), with some modifications. Petri dishes (100-mm) were filled to a depth of 5 mm with GC agar, stored in a refrigerator, and used within 5 days of preparation. Plates were dried for 30 min before use. The gonococcal suspension was prepared as described above. By using a cotton swab from which excess suspension had been removed by rotating the swab against the side of the tube, the suspension was streaked evenly in three planes onto the surface of the medium. After allowing 5 to 15 min for drying, sensitivity disks were placed on the surface of the plates. The plates were incubated, without delay, at 35 C under 5% carbon dioxide. At 18 to 24 h, the diameter of the zone of inhibition which had formed around the disk was measured with calipers to the nearest millimeter. The zones of inhibition which formed were clear and unambiguous to the naked eye.

**Data analysis.** For each antibiotic, the results of agar dilution (y axis) and disk diffusion (x axis) tests were plotted on a scatter graph. The MIC was plotted on a log<sub>2</sub> scale. A relative MIC scale was assigned for each graph and used in the statistical calculations. The zone of inhibition was plotted on a linear scale as the radius squared. The log of the MIC was found to be more closely related to the radius squared than to the diameter of the inhibition zone. The theoretical basis for this is described in detail by Cooper (3). The corresponding zone diameter is given on a second scale. The least squares line of regression (y on x), and correlation coefficient (r) were calculated by using a Wang 600 series programmed calculator. The equation for the regression line is given as  $y = bx + a$ , where  $a$  is the y intercept expressed as the relative MIC and  $b$  is the slope.

## RESULTS

The susceptibility of a large number of gonococcal isolates to penicillin, ampicillin, tetracycline, erythromycin, cephaloridine, and chloramphenicol was determined by agar dilution (MIC) and disk diffusion techniques. With all antibiotics, the isolates tested covered essentially the full range of susceptibility observed at the Philadelphia Health Laboratories during 1971 and 1972. Results of MIC and disk testing with the six antibiotics are shown in Fig. 1 to 6.

MIC and disk results were found to correlate well with each of the antibiotics over the full range of susceptibility. The coefficient of correlation ranged from -0.79 with cephaloridine to -0.93 with penicillin. The high coefficients of correlation obtained between MIC and disk methods show that the results of disk testing are of significant value in predicting susceptibility of isolates of *N. gonorrhoeae*.

In order to show the reproducibility of the disk method, three isolates with markedly different degrees of susceptibility were tested in duplicate on three different days with each of the six antibiotics. As shown in Table 1, the diameter of the zone of inhibition was reproducible within  $\pm 2$  mm (with one exception) at the 95% confidence level.

A difference in inoculum size is one of the most important variables which may influence the results of disk susceptibility testing from day to day. The size of the inoculum was varied from  $1 \times 10^4$  to  $5 \times 10^8$  colony-forming units per ml (Table 2). With both penicillin and tetracycline, a 10-fold variation in inoculum size produced a change of about 4 mm in the diameter of the inhibition zone.

## DISCUSSION

We have described a disk diffusion procedure suitable for testing the susceptibility of gonococcal isolates to a variety of antibiotics. Some modification of the procedure described by Bauer et al. (1), which has been widely used with many fast-growing pathogens, was required. GC agar, as described, was used in place of Mueller-Hinton agar. In the Bauer-Kirby system the inoculum is prepared from broth cultures. With gonococci, an inoculum prepared by suspending 18- to 24-h colonies obtained from GC agar was found to be satisfactory. The suspension was standardized and inoculated as described for the Bauer-Kirby system, and the same antibiotic disks were used. Plates were incubated at 35 C under 5% carbon dioxide, and disk zone sizes were read after 18 h incubation.

At present, the USPHS recommends four antibiotics for use in the treatment of uncomplicated gonorrhea (2). The recommended treatment of choice is 4.8 million units of aqueous procaine penicillin G or 3.5 g of ampicillin, in both cases accompanied by 1.0 g of probenecid. When either regimen is contraindicated, the alternative treatment is 2 to 4 g of spectinomycin or 1.5 g of tetracycline in an initial dose followed by 0.5 g four times a day until a total dose of 9 g is administered. The dosages recommended are close to the max-

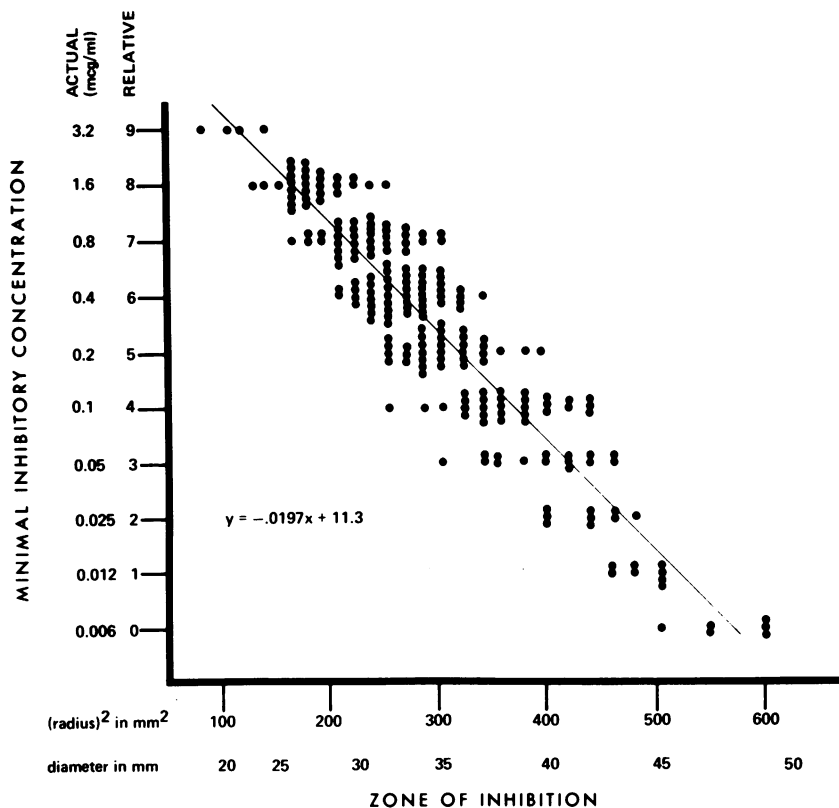


FIG. 1. Susceptibility of 225 gonococcal isolates to penicillin as determined by agar dilution and disk diffusion methods. The coefficient of correlation is  $-0.93$ . In the equation for the regression line,  $y$  is the relative MIC and  $x$  is the (radius)<sup>2</sup> of the inhibition zone.

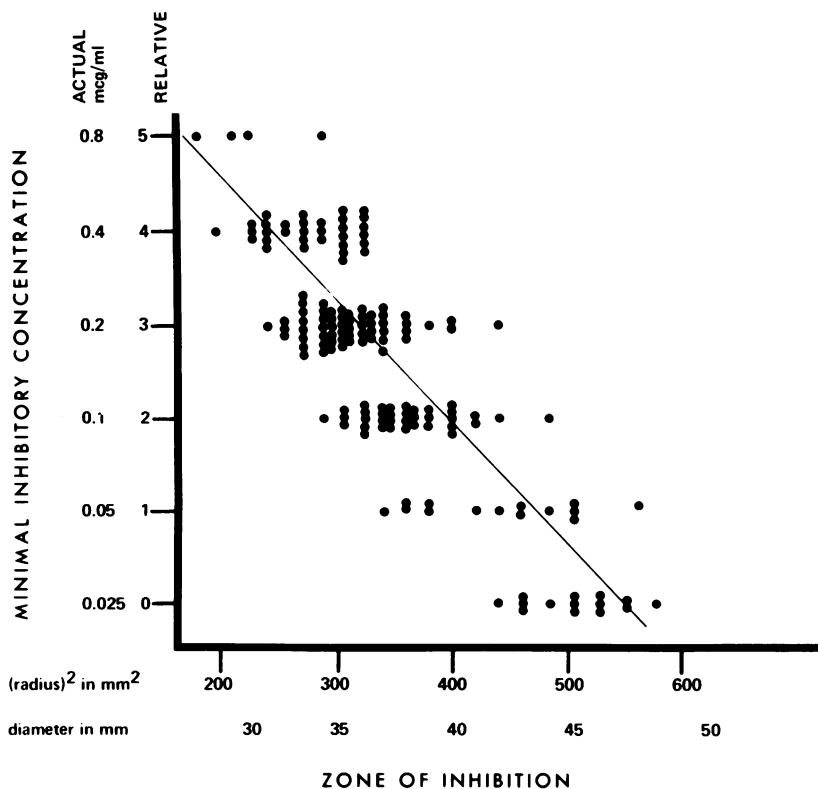
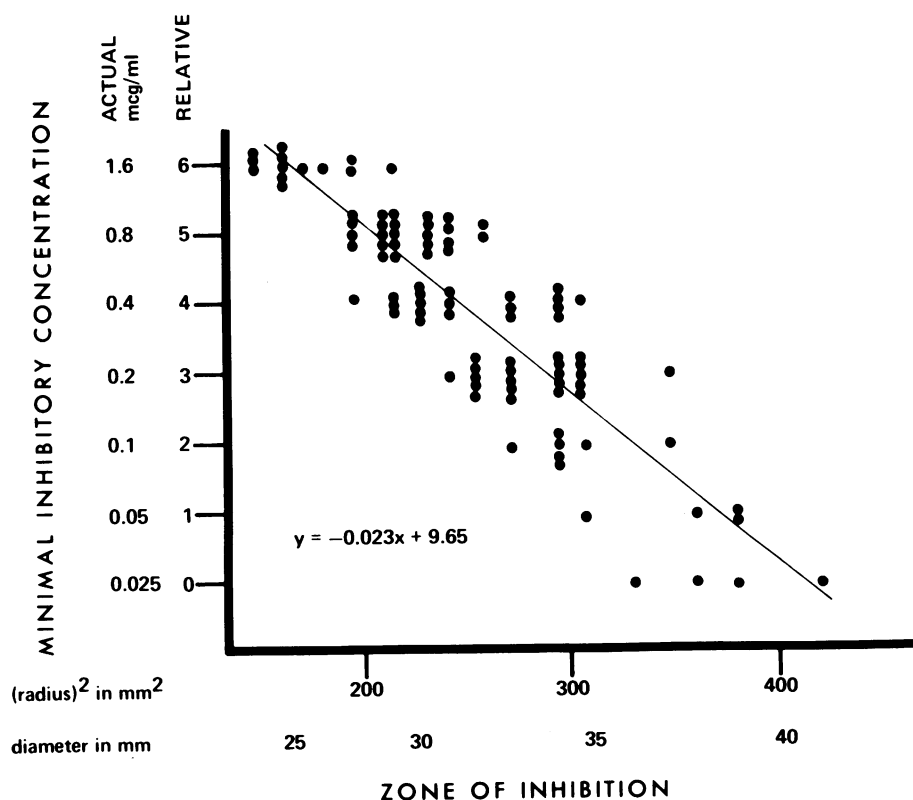
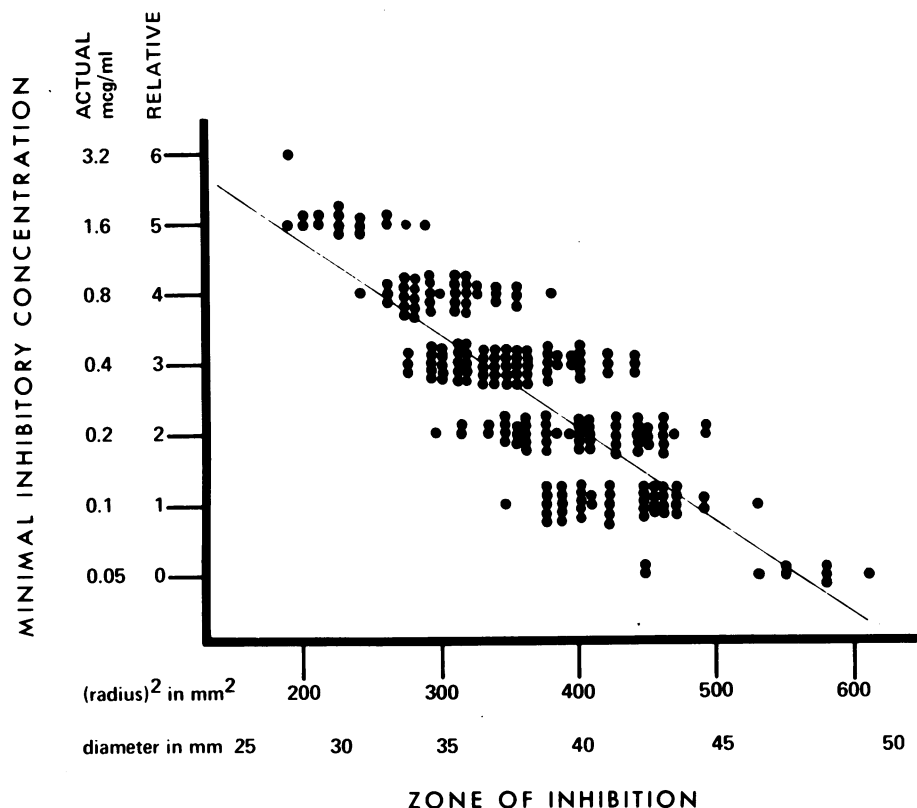


FIG. 2. Susceptibility of 160 gonococcal isolates to ampicillin as determined by agar dilution and disk diffusion methods. The coefficient of correlation is  $-0.84$ . In the equation for the regression line ( $y = -0.0126x + 6.92$ ),  $y$  is the relative MIC and  $x$  is the (radius)<sup>2</sup> of the inhibition zone.



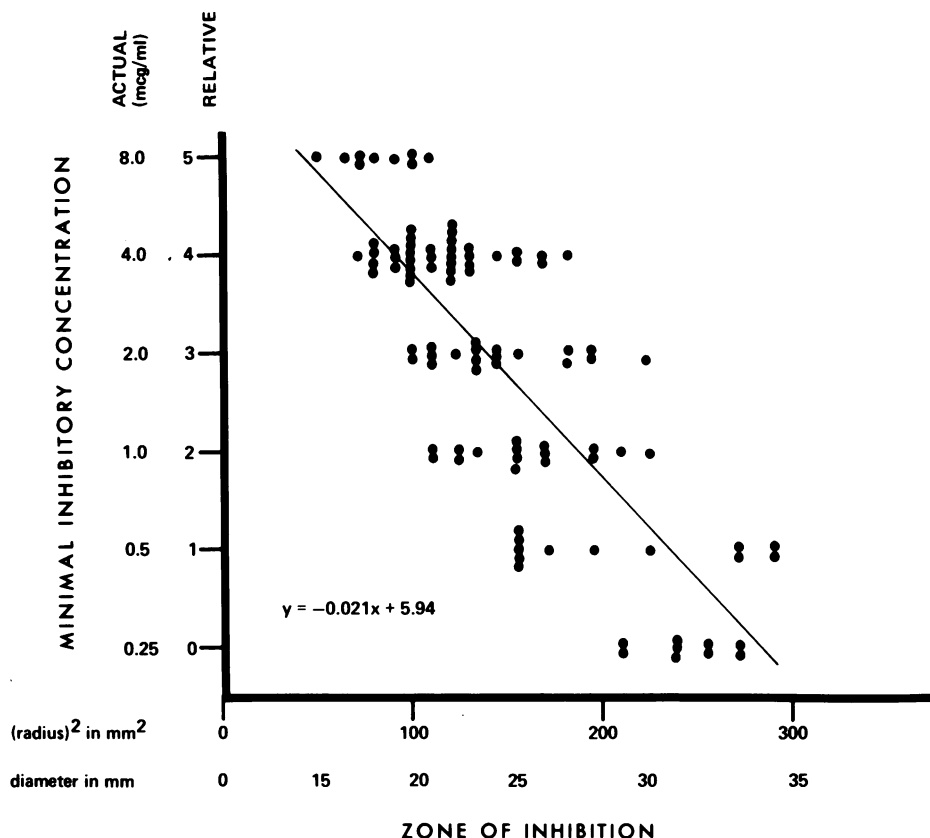


FIG. 5. Susceptibility of 101 gonococcal isolates to cephaloridine as determined by agar dilution and disk diffusion methods. The coefficient of correlation is  $-0.79$ . In the equation for the regression line,  $y$  is the relative MIC and  $x$  is the  $(\text{radius})^2$  of the inhibition zone.

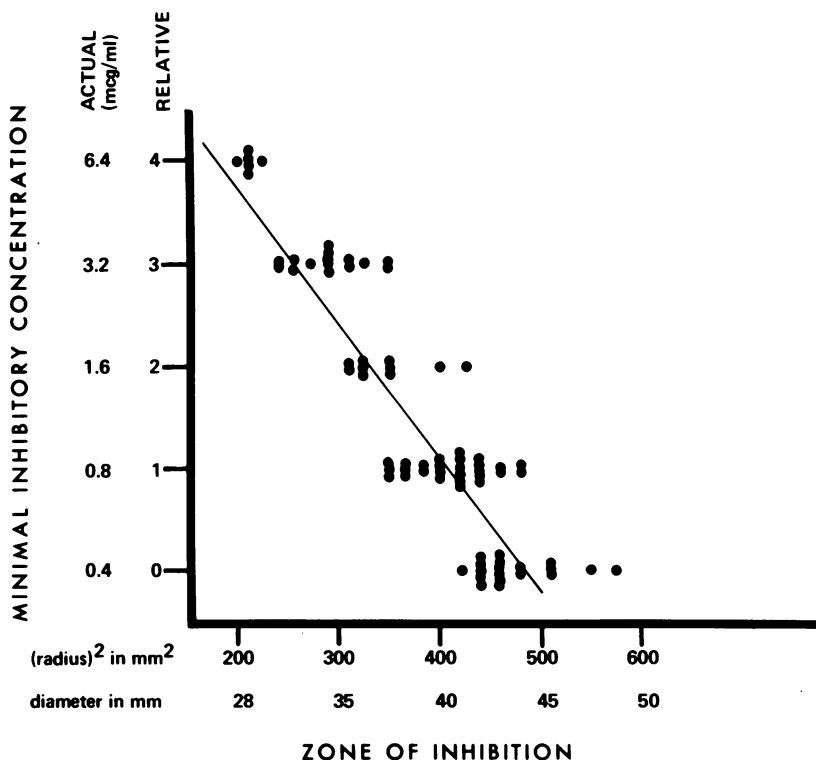


FIG. 6. Susceptibility of 78 gonococcal isolates to chloramphenicol as determined by agar dilution and disk diffusion methods. The coefficient of correlations is  $-0.91$ . In the equation for the regression line ( $y = -0.0128x + 6.37$ ),  $y$  is the relative MIC and  $x$  is the  $(\text{radius})^2$  of the inhibition zone.

TABLE 1. *Reproducibility<sup>a</sup> of disk test with three strains of Neisseria gonorrhoeae*

Antibiotic	Average zone diameter $\pm$ two standard deviations		
	Strain S7-13	Strain 49191	Strain S7-14
Penicillin	30.2 $\pm$ 1.2	42.4 $\pm$ 1.5	52.9 $\pm$ 0.7
Ampicillin	31.9 $\pm$ 1.4	36.7 $\pm$ 1.9	44.8 $\pm$ 0.5
Tetracycline	27.7 $\pm$ 1.4	32.7 $\pm$ 1.6	37.2 $\pm$ 0.7
Erythromycin	30.9 $\pm$ 0.7	40.3 $\pm$ 1.2	50.9 $\pm$ 1.4
Chloramphenicol	12.9 $\pm$ 4.0	24.4 $\pm$ 1.0	32.1 $\pm$ 0.4
Cephalexidine	35.1 $\pm$ 0.6	41.5 $\pm$ 1.6	52.5 $\pm$ 0.5

<sup>a</sup> Results of tests performed in duplicate on three different days.

imum that can be administered on an outpatient basis (10, 11). As discussed below, recent studies which have examined the efficacy of the currently recommended treatment schedules have observed cure rates approaching 100%. Lower dosages have been shown to result in a significant number of treatment failures (13, 14, 16).

As discussed in a review by Sparling (24), many studies have shown that high doses of penicillin or ampicillin, combined with probenecid, will produce high cure rates despite the frequent appearance of gonococcal isolates resistant to 0.5  $\mu$ g of penicillin (8, 9, 11, 12, 15) per ml. In 1967, Holmes et al. (9) reported no treatment failures in a group of patients treated with the presently recommended regimen of tetracycline. Recently, Holmes et al. (10) reported a cure rate of 98.2% for men and 96.3% for women treated respectively with either 2.4 or 4.8 million units of aqueous procaine penicillin G plus probenecid. Almost 20% of gonococci encountered in this study required 1.0  $\mu$ g of penicillin per ml for inhibition of growth in vitro. In addition, Johnson et al. (11) observed no treatment failures in groups of patients treated with either 6 million units of crystalline procaine penicillin combined with probenecid, 3.5 g of ampicillin plus probenecid, or 2.5 g of tetracycline. The most resistant gonococci encountered in these studies exhibited MICs of 0.8  $\mu$ g of penicillin per ml, 0.6  $\mu$ g of ampicillin per ml, and 2.0  $\mu$ g of tetracycline per ml.

It is appropriate to ask what is the role of antibiotic susceptibility testing of gonococci in the clinical laboratory. By using the disk technique, the clinical laboratory can determine the approximate MIC of any strain of *N. gonorrhoeae* to a number of antibiotics. This information can be used in evaluating an apparent treatment failure and in determining the appropriate antibiotic to be used in retreatment. This evaluation would depend upon the

correlation between treatment failure, the MIC of the infecting organism, and the particular dose of antibiotic used. It would also be feasible to establish directly the relationship between disk results and treatment failure. With respect to the currently recommended treatment schedules for penicillin, ampicillin, and tetracycline, it is not yet certain what level of gonococcal resistance is clinically significant. However, there is some information upon which to base a reasonable estimate.

Those gonococci which require 2 to 4  $\mu$ g of tetracycline per ml for inhibition can be correlated with treatment failure to the currently recommended schedule of tetracycline (W. W. Karney, personal communication). With penicillin G, a significant percentage of treatment failures has been reported by Niordson and Ullman (19) after treatment with 5 million units of aqueous penicillin G plus probenecid. Failures were associated with infections by gonococci which required more than 2.7  $\mu$ g of penicillin per ml for inhibition (19, 24). It is not known whether gonococcal isolates resistant to 2.7  $\mu$ g of ampicillin per ml would yield a significant number of failures after treatment by the USPHS-recommended 3.5 g of ampicillin plus probenecid. Comparable concentrations of ampicillin and penicillin in serum are achieved by the respective recommended dosages (11), but a lower concentration of ampicillin than penicillin is required in vitro to inhibit the more penicillin-resistant gonococci (5). These facts

TABLE 2. *Effect of varying the inoculum on the zone diameter around penicillin and tetracycline disks as determined with seven different gonococcal isolates<sup>a</sup>*

Gonococcal isolate number	Standard zone diameter around penicillin disk	Change in zone diameter around penicillin disk per 10-fold change in inoculum concn	Standard zone diameter around tetracycline disk	Change in zone diameter around tetracycline disk per 10-fold change in inoculum concn
1	28	3.2	35	3.5
2	33	4.4	34	4.0
3	30	4.4	35	4.0
4	41	5.8	38	2.7
5	39	5.2	39	3.4
6	41	3.0	35	3.6
7	34	5.5	33	4.4
		Avg 4.5 $\pm$ 1.1 SD		Avg 3.7 $\pm$ 0.55 SD

<sup>a</sup> The concentration of inoculating suspension was varied between  $1 \times 10^4$  and  $5 \times 10^8$  colony-forming units per ml. Values are expressed in millimeters. SD, standard deviation.

suggest that gonococci resistant to 2.7  $\mu\text{g}$  of ampicillin per ml can, at times, still be cured by ampicillin.

At present, however, the majority of gonococcal isolates encountered in the United States requires significantly less than 2.7  $\mu\text{g}$  of either penicillin or ampicillin per ml or 4.0  $\mu\text{g}$  of tetracycline per ml for inhibition. Moreover, at least in some locations, no isolates are encountered which require more than 1.0  $\mu\text{g}$  of penicillin, ampicillin, or tetracycline per ml (7).

The results of the studies reported here, as well as earlier studies (21, 23), show the feasibility of using a disk susceptibility test with gonococci. The results with such a test can be reproducible and may correlate well with results obtained by an MIC test. Apparently clinical susceptibility testing of gonococci is not widely practiced now.

#### ACKNOWLEDGMENTS

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